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14. ABSTRACT Twenty-five multi-drug resistant organisms (MDROs) of high military importance were sequenced using the Illumina HiSeq platform to identify drug resistance and virulence mechanisms contributing to their success as pathogens in the wound environment. The genomes were annotated and submitted to GenBank. Novel bioinformatics pipelines have been developed to (1) identify highly variable genomic regions, (2) classify antibiotic resistance islands, and (3) determine diversity of gene clusters associated with virulence in <i>A. baumannii</i> . Three top priority MDROs and three commensal microbes were chosen and obtained from WRAIR MRSN and ATCC for <i>in vitro</i> co-culturing in confrontation assays aimed at investigating alterations in gene expression, particularly antibiotic resistance genes and virulence factors. Confrontation assays were also carried out to assess gene regulation when the MDROs were co-cultured with cultured human skin cells. RNA-seq analysis of the bacteria-bacteria and bacteria-human confrontation assays are currently in progress. Manuscripts are in preparation describing the findings from this study.					
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INTRODUCTION

Antimicrobial resistance in bacterial pathogens is steadily increasing and recognized as one of the greatest threats to global public health. This is of particular concern to the Military Health System since recent wars in Afghanistan and Iraq resulted in a major spike in the number of wound and healthcare-acquired infections by bacteria resistant to three or more antibiotics (i.e., multi-drug resistant organisms, or MDROs). This study uses advanced genomic and bioinformatics approaches coupled with cell culturing to identify and characterize factors that influence virulence, including antibiotic resistance and biofilm formation in MDROs. This objective will be accomplished through experiments focusing on the regulatory responses of both target and challenger organisms in bacterial-bacterial and bacterial-eukaryotic interactions likely to occur in the wound environment. Deep next-generation cDNA sequencing is being used as a tool to dissect the regulatory networks altered during these organismal-level confrontations. Cell-cell challenges include: MDROs versus human microbiome organisms, MDROs versus other MDROs and MDROs versus the human host via cell culture. It is anticipated that there will be specific interacting partners that have a negative effect on MDRO virulence, while other interactors will induce the expression of genes involved in virulence. This project is being carried out in collaboration with the Walter Reed Army Institute of Research Multidrug-resistant Organism Repository and Surveillance Network (WRAIR MRSN), who have provided isolates of highest clinical importance collected from military health care facilities. The research outcome may lead to novel treatment of combat wound infections in the future.

BODY

Progress during the second year of the project period has been focused on fulfilling the goals outlined in the proposed scope of work by 1) performing high-level bioinformatics analysis of the MRSN genomes sequenced in this study to identify genomic determinants related to pathogenesis and antibiotic resistance in *A. baumannii*, 2) analyzing differential gene expression profiles of bacteria-bacteria confrontation assays between MDROs and skin commensal bacteria, and 3) conducting confrontation assays between MDROs and human cells to determine gene regulation in association with bacterial interactions with cultured human skin cells. Each of these will be discussed in detail under each aim of the scope of work.

Aim 1a. Sequence characterization of 25 MDROs from WRAIR MRSN

We have completed genome sequencing and assembly of 25 high priority clinically relevant MDRO isolates provided by MRSN. The genome assembly and annotation have been submitted to GenBank and also shared with MRSN collaborators.

In addition to performing genome annotation using the JCVI annotation pipeline, we have performed higher-level comparative analysis of genomic features including flexible genomic islands (fGIs), antibiotic resistance islands (RIs), and virulence factors in the seven *A. baumannii* genomes sequenced in this study in comparison to 242 publicly available *A. baumannii* genomes.

Flexible genomic region (fGR) analysis

Differences among bacterial strains tend to be mobile elements (e.g., prophage, plasmids, integrated elements) and genes encoding for O-antigen or capsular polysaccharides (CPS)[1-6]. These “flexible” regions also encode genes involved in cell surface structures (i.e., O-antigen, CPS, teichoic acid, S-layer, flagella, pili, and porins) as well as genes for resource utilization. These regions that vary between strains have been referred to as flexible genomic islands (fGIs)[7]. We define flexible genomic regions (fGRs) as locations between sets of adjacent core genes of the consensus core pan-genome. fGRs are made up of multiple fGIs, which are syntenic clusters of genes that are not part of the core and tend to be strain-specific. To identify the core and fGRs of the *A. baumannii* chromosome, we implemented a novel algorithm to produce a consensus pan-genome based on orthologous protein clusters produced from the JCVI Pan-genome Ortholog Clustering Tool, PanOCT [8]. The frequency and length of the fGRs are shown in Figure 1. For example, features “K” and “O” at 0.1 Mb and 4.4 Mbp correspond to the K and O antigen loci respectively. The location is conserved, but there are different instances or alleles (i.e., fGIs) of each of these loci in different strains of *A. baumannii*. Feature “1” at 0.3 Mbp corresponds to the *comM* gene locus often found inserted with antibiotic resistance islands among *A. baumannii* isolates.

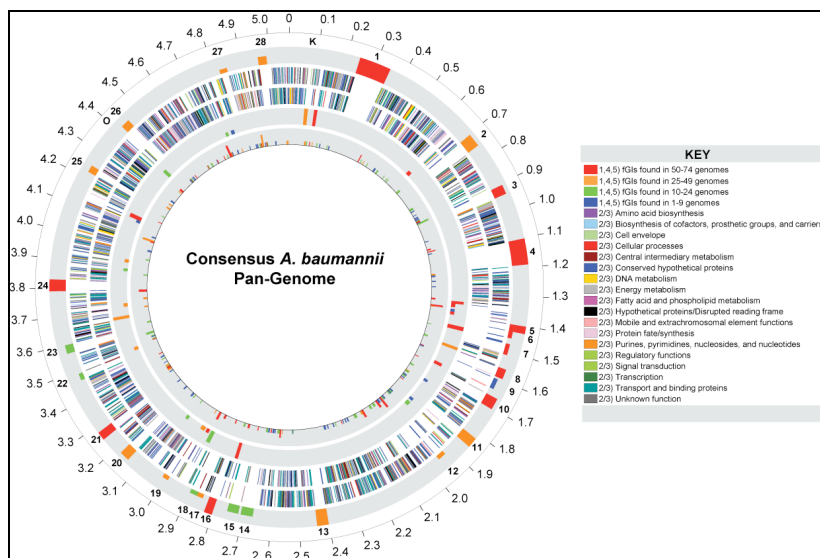


Figure 1. Consensus *A. baumannii* Pan-Genome. The *A. baumannii* pan-genome is depicted as a circle where each concentric circle numbered from the outermost to the innermost circle. Circles 2 and 3 represent the core pan-genome, while circles 4, and 5 represent regions between core genes containing fGIs. Circles 1, 4 and 5 depict fGIs >20, >10-20, and 1-10 kb in length, respectively. The K and O antigen loci are noted and fGIs > 20 kb are numbered around the circumference of the outer circle. The height of fGI bars describes the number of fGIs per region: quarter height (1-3 fGIs), half height (4-6 fGIs), three quarter height (7-9 fGIs), and full height (10+ fGIs). The key describes color representations for each circle. Colors for the core genes are functional main role categories while colors for fGIs denote the number of genomes the fGIs are found in.

Diversity of resistance islands and virulence features

In addition to the fGI analysis, we have also undertaken targeted approaches to classify antibiotic resistance signatures and virulence factor determinants among the seven MRSN *A. baumannii* isolates. A summary of the analyses is shown in Table 1. We defined RI signatures based on both the genomic location and type of RI insertion found in a given genome. AbaR3 and 4 RIs were identified at the *comM* insertion hot spot across six of the MRSN isolates. Interestingly, MRSN 7339 carried a secondary RI insertion at the *pho* location in addition to *comM*. This is the first example of dual RI insertions found at both the *comM* and *pho* location since previously reported in the reference strain AB0057/2004 [9].

We have also determined the distribution and conservation of gene clusters that encode virulence factors involved in adherence, iron uptake, efflux, biofilm formation, etc. With regard to iron sequestration, in general *A. baumannii* carries two siderophore clusters, one of which is the *Acinetobacter*-specific acinetobactin system. Among the MRSN isolates, we have unexpectedly identified a third cluster (cluster 2), which is thought to be rare. Cluster 2 was only previously detected exclusively in the *A. baumannii* isolate ATCC 17978 and no other isolates analyzed to-date [10]. We have identified cluster 2 in four MRSN isolates, which belong to ST81 and 94. Furthermore, all four cluster 2-positive strains were isolated in 2011 sourced from wound samples originating from the US military healthcare system. Future functional studies will be needed to characterize the type of siderophore and associated iron affinity produced from this cluster.

Table 1. Distribution of siderophore gene clusters and resistance island signatures across MRSN *A. baumannii* isolates sequenced in this study.

Isolates	Genome category	Source	Year	ST	Allele summary	Country	Siderophore				Resistance islands
							Cluster 1	Cluster 2	Cluster 3 (Acinetobactin)	Cluster 4	
AYE	Reference	Urinary	2001	1	1-1-1-1-5-1-1	France	+	-	+	-	comM/AbaR1
ACICU	Reference	Internal	2005	2	2-2-2-2-2-2-2	Italy	+	-	+	-	comM/AbaR2
ATCC 17978	Reference	Misc.	1951	novel	3-2-2-2-30-4-28	n.a.	+	+	+	-	
SDF	Reference	Misc.	2000	17	3-29-30-1-9-1-4	France	-	-	-	-	
WC487	Acinetobacter sp.	n.a.	n.a.	n.a.	n.a.	n.a.	-	-	-	+	
MRSN 3527	MRSN	Wound	2011	81	1-1-1-1-5-1-2	USA	+	+	+	-	comM/AbaR4
MRSN 3405	MRSN	Wound	2011	94	1-2-2-1-5-1-1	USA	+	+	+	-	comM/AbaR4
MRSN 3942	MRSN	Wound	2011	94	1-2-2-1-5-1-1	USA	+	+	+	-	comM/AbaR4
MRSN 4106	MRSN	Wound	2011	94	1-2-2-1-5-1-1	USA	+	+	+	-	comM/AbaR4
MRSN 7339	MRSN	Wound	2004	1	1-1-1-1-5-1-1	USA	+	-	+	-	comM/AbaR3 + pho
MRSN 58	MRSN	Wound	2010	1	1-1-1-1-5-1-1	USA	+	-	+	-	comM/AbaR3
MRSN 7341	MRSN	Respiratory	2004	2	2-2-2-2-2-2-2	USA	+	-	+	-	comM/other

Aim 1b. Perform bacterial-bacterial confrontation assays to monitor bacterial cell interactions between MDROs and the human skin microbiome, and interactions between MDROs.

Three MDROs (*Acinetobacter baumannii* MRSN 7339, *Klebsiella pneumoniae* MRSN 1319, and *Enterobacter cloacae* MRSN 11489) and three commensal organisms (*Staphylococcus epidermidis* SK-135, *Lactobacillus reuteri* SD2112 ATCC 55730 and *Corynebacterium jeikeium* ATCC 43734) were used to set up pair-wise confrontation assays as shown in **Table 2**. Illumina RNA-seq library construction and HiSeq sequencing have been completed for all bacterial-bacterial confrontations.

Table 2. Bacteria-Bacteria Confrontation Assays

Target Species		Commensal Controls	Challenger species		
			<i>E. cloacae</i>	<i>K. pneumoniae</i>	<i>A. baumannii</i>
			EC	KP	AB
<i>S. epidermidis</i>	SE	SE:SE	EC:SE	KP:SE	AB:SE
<i>L. reuteri</i>	LR	LR:LR	EC:LR	KP:LR	AB:LR
<i>C. jeikeium</i>	CJ	CJ:CJ	EC:CJ	KP:CJ	AB:CJ
<i>E. cloacae</i>	EC		EC:EC	KP:EC	AB:EC
<i>K. pneumoniae</i>	KP			KP:KP	AB:KP
<i>A. baumannii</i>	AB				AB:AB

Gene expression analysis of *A. baumannii* with other MDROs or commensals

(1) Up-regulation of *A. baumannii* genes when co-cultured with *K. pneumoniae*, *E. cloacae* or *C. jeikeium*. *A. baumannii* up-regulated some genes in response to all three confrontations and some specifically to each species tested (Figure 2).

Among the uniquely up-regulated *A. baumannii* genes specific to the confrontation with the MDRO *K. pneumoniae*, fGI classified genes were identified, of which three were phage associated, one was the β -lactamase in the *comM* fGI region, and the remaining were hypothetical or unclassified with one protein identified as the carbon starvation protein CstA. Other uniquely up-regulated genes include genes associated cell envelope, other cellular processes, energy metabolism and one Ton-B-dependent siderophore receptor, efflux and ABC type transporters and hypothetical proteins.

The 35 uniquely up-regulated *A. baumannii* genes specific to confrontation with *E. cloacae* include: fGI genes that appear to be involved in energy metabolism (succinylornithine transaminase/acetylornithine aminotransferase domain protein and arginine N-succinyltransferase), transport and binding family proteins that include Ton-B dependent receptors, ABC transporters and some iron-chelate and siderophore interacting proteins.

The greatest number of differentially regulated genes in any confrontation was observed when *A. baumannii* was co-cultured with the commensal *C. jeikeium*, where 185 *A. baumannii* genes were differentially expressed, of which 141 genes were unique to this confrontation. About half of these are categorized as hypothetical proteins or have not been assigned functions, but in contrast to the MDRO-MDRO confrontations, we observed specific up-regulation of regulatory functions and cell envelope related functions. More fGI and fGI phage genes were also differentially expressed in this particular co-culture. Most of these were hypothetical proteins, but also includes a cold shock protein CspE and a CRISPR-associated helicase. One distinct phenomenon was the up-regulation of Type IV pili genes only observed in the *A. baumannii* - *C. jeikeium* confrontation.

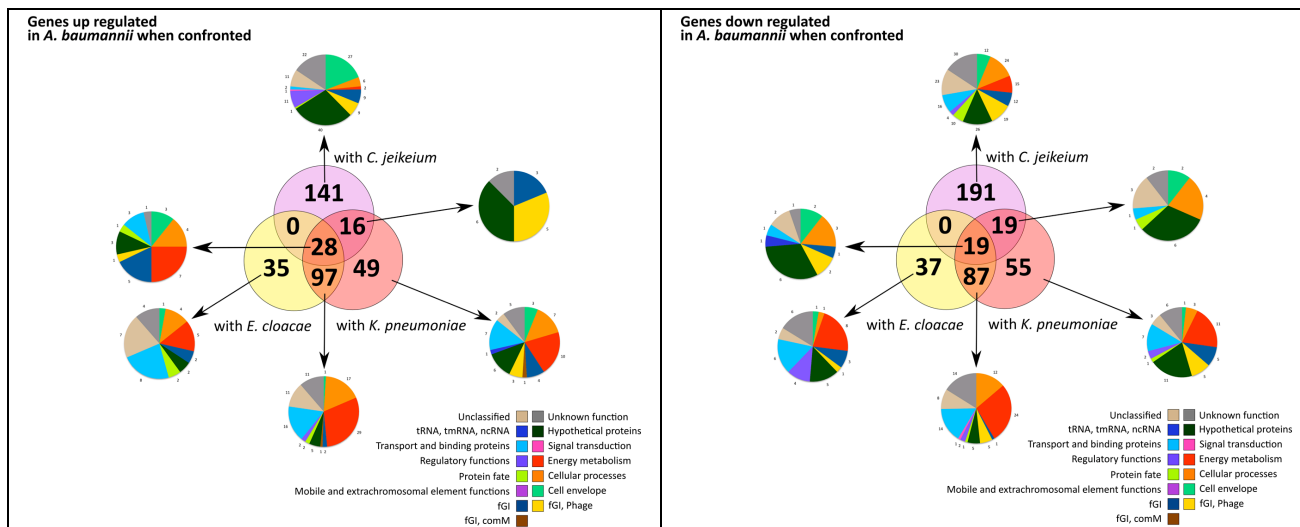


Figure 2. Up- or down-regulated genes in *A. baumannii* when confronted with other MDROs or commensal bacteria.

(2) Down-regulation of *A. baumannii* genes when co-cultured with *K. pneumoniae*, *E. cloacae* or *C. jeikeium*. Only 19 genes were commonly down-regulated when *A. baumannii* confronted each of the other MDROs and the commensal (Figure 2, right panel). There was no striking functional classes of genes that were down-regulated in response to *K. pneumoniae*, which included some fGI genes, hypothetical proteins, genes of unknown function or unclassified, energy metabolism, transport and binding. Similarly, when in co-culture with *E. cloacae*, there was no distinctive pattern of down-regulation of a particular functional category. In both these cases, the energy metabolism genes that were down-regulated appear to be those that would not be beneficial to the particular environment, unlike the metabolic pathways that could be induced for competitive growth and survival against competing bacteria under limited nutrient conditions. Similar to the trend of upregulated genes of *A. baumannii* in response to *C. jeikeium*, 229 genes were down-regulated, of which almost all (191) were specific to this confrontation. Several of these genes encode hypothetical proteins, or were unclassified or had unknown function, but we also observed an increased number of genes involved in protein transport and processing, cell envelope related functions, transport and binding, and those categorized as involved in cellular processes. The latter category encoded proteins like CsuE, universal stress proteins and a β -lactamase.

(3) Validation of RNA-Seq with qPCR. RNA-seq data for the bacterial confrontations was validated through qPCR of select differentially expressed genes. Biological replicates were both tested and qPCR was performed using cDNA obtained from reverse transcription of total RNA. qPCR reactions were performed in triplicate using SYBR Green master mix from Roche on a LightCycler480 qPCR machine. For *K. pneumoniae*, two down-regulated and 3 up-regulated genes were tested. For *A. baumannii* three down regulated and 4 up regulated genes were tested. Relative expression ratios were determined for genes of interest by normalizing to the housekeeping gene *gyrB* in the monoculture controls. Relative expression ratios were converted to log fold change (logFC) and compared to the logFC from RNA-seq data. All genes tested showed the same direction of logFC in both RNA-Seq and qPCR analyses (Figure 3).

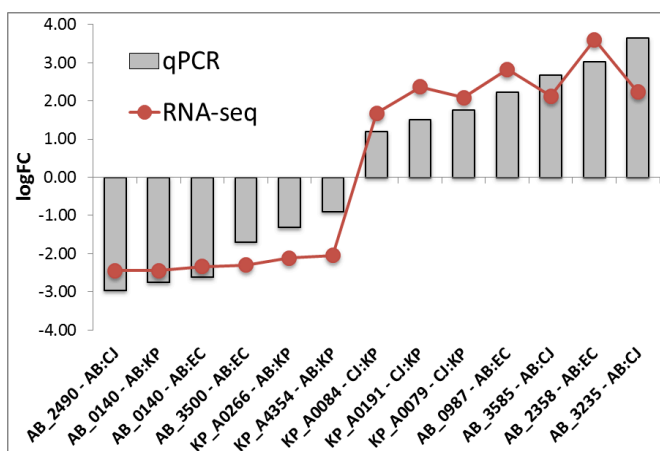


Figure 4. qPCR analysis to confirm differential gene expression observed in RNA-seq.

Aim 2. Perform bacterial-eukaryotic confrontation assays to monitor bacterial-host cell interactions between MDROs and human skin cell cultures.

Confrontations of the three MDRO isolates with human dermal fibroblasts (HDF) cells and self-controls have been completed in duplicate (MOI and time of incubation noted below). MiSeq data was analyzed to confirm successful rRNA depletion and that the libraries contain reads of expected bacterial or human origins (Table 3).

Table 3. Mapping results of MiSeq reads for the bacteria-human confrontation assays

Samples	Total reads	Mappable reads				Percentage reads mapped			
		AB	KP	EC	Human	AB	KP	EC	Human
AB-1	4,188,432	3,395,759	13,212	2,652	4,066	81.1%	0.3%	0.1%	0.1%
AB-2	1,540,914	1,160,679	4,802	971	9,937	75.3%	0.3%	0.1%	0.6%
KP-1	1,637,180	6,302	1,215,961	142,017	5,912	0.4%	74.3%	8.7%	0.4%
KP-2	1,936,942	7,623	1,527,390	185,352	2,500	0.4%	78.9%	9.6%	0.1%
EC-1	1,843,764	4,992	189,247	1,371,173	6,484	0.3%	10.3%	74.4%	0.4%
EC-2	1,906,378	6,593	210,965	1,493,238	5,082	0.3%	11.1%	78.3%	0.3%
HDF-1	1,541,534	353	590	276	1,272,210	0.0%	0.0%	0.0%	82.5%
HDF-2	2,003,470	321	430	221	1,765,590	0.0%	0.0%	0.0%	88.1%
AB-HDF-1	1,636,280	510,653	2,143	659	814,921	31.2%	0.1%	0.0%	49.8%
AB-HDF-2	1,724,904	556,499	5,547	1,047	951,086	32.3%	0.3%	0.1%	55.1%
KP-HDF-1	2,417,894	6,224	1,047,908	100,437	859,218	0.3%	43.3%	4.2%	35.5%
KP-HDF-2	2,086,710	2,901	583,987	58,635	1,141,162	0.1%	28.0%	2.8%	54.7%
EC-HDF-1	436,086	259	1,103	5,943	84,587	0.1%	0.3%	1.4%	19.4%
EC-HDF-2	3,302,168	6,077	111,678	1,081,905	1,781,741	0.2%	3.4%	32.8%	54.0%

Subtask. Carry out pilot experiments to determine sub-lethal infection (multiplicity) ratio of bacterial and human cells for co-culture experiments.

HDF cell cultures were set up to test multiplicity of infection (MOI) as well as incubation times for individual MDROs. The MOIs for all three MDROs with HDF cells was determined to be optimal at 100:1 (MDRO:HDF). The length of confrontation was tested whereby one-hour duration was determined to be optimal. Prolonged confrontation times resulted in acidification of the cell culture media, which was avoided to eliminate interference with gene expression due to pH changes and direct interactions among the MDROs.

Subtask. Determine that bacterial isolates adhere to human cells prior to isolation of RNA.

Bacterial retention control experiments were repeated and the MDROs were observed via light microscopy to be adherent to the HDF cells, even after 3 rinses with PBS (data not shown). Cells were isolated for RNA extraction from confrontations after removing excess culture media so only adherent/close proximity bacterial cells were harvested. Cells were then treated with RNaprotect Cell Reagent (Qiagen) to immediately stabilize RNA transcripts before further processing.

To-be completed tasks during the 6 month no-cost extension period.

Task 3c. Bacterial-host RNA-Seq and gene expression analysis. *Isolate human polyA mRNA from samples to collect transcripts derived from human skin cells. Isolate bacterial mRNA from samples by depletion of human mRNA and bacterial and eukaryotic rRNAs to collect transcripts derived from the MDRO isolates. Combine bacterial and human mRNA, and construct barcoded ssRNA-Seq libraries for Illumina sequencing. De-multiplex Illumina reads and carry out de novo and reference-based transcript assembly to reconstruct gene expression levels in the bacterial and human skin cell transcriptomes. Analyze genome-wide differential gene expression levels with a focus on genes involved in antibiotic resistance in the MDRO microbes and innate defense immune system in the human skin cells. Share analyzed results with WRAIR and publish research outcomes in presentations and peer-reviewed publications.*

The set of bacterial-human confrontation libraries has passed library quality and contamination checks by MiSeq sequencing, and has been sequenced on four lanes of 2x100 bp Illumina HiSeq 2000. Expression analysis on the bacteria and human gene sets will be carried out during the no-cost extension period.

KEY RESEARCH ACCOMPLISHMENTS

- Completed genome sequencing and annotation of 25 MDROs of military importance
- Developed a graph-based algorithm to identify highly variable genomic regions (fGIs) across a given large group of bacterial isolates
- Developed bioinformatics approaches for characterizing the diversity of RIs and virulence factors in *A. baumannii*
- Analyzed gene expression profiles in *in vitro* confrontation assays between selected MDROs and skin commensals
- Conducted confrontation assays between selected MDROs and cultured adult human dermal fibroblasts

REPORTABLE OUTCOMES

- A set of annotated genomes of 25 MDROs of military importance submitted to NCBI GenBank and shared with MRSN collaborators.
- A set of highly variable genomic regions including K and O antigen loci, which control virulence and antigenicity, were identified based on analysis across over 240 military and civilian *A. baumannii* isolates.
- A comprehensive catalog of genomic features representing RIs and virulence diversity for seven *A. baumannii* strains isolated from US military personnel and provided by WRAIR MRSN.
- A group of differentially regulated genes when MDROs are in confrontation with other MDROs or commensal bacteria.

CONCLUSION

Our project goal is to identify and characterize factors that influence virulence, including antibiotic resistance, and biofilm formation in MDROs. Of particular interest are microbial or host-derived signals that induce the expression of virulence-associated genes. This knowledge will enhance the detection, characterization, and reporting of multidrug-resistant organisms in the Military Health System. This research will ultimately enhance the knowledge products the MRSN provides to its customers and stakeholders, and may lead to future drugs to be used in conjunction with antibiotic therapies that prevent the expression of drug resistance genes.

To date, we have sequenced and annotated the genomes of 25 MDROs of military importance. We have carried out confrontation assays to study gene regulation by MDROs in human skin commensal microbes and cell cultures. Gene expression analysis by RNA-seq, with a focus on the expression of virulence factors, are being carried out for the bacteria-bacteria confrontation assays. The bacteria-human confrontation assays have been performed and gene expression analysis will be carried out during the no-cost extension period.

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